

**Gene expression profile of human cytokines in response to
B.pseudomallei infection**

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ABSTRACT

Melioidosis, is an under-reported infectious disease, caused by the gram-negative bacterium *Burkholderia pseudomallei*. Understanding disease pathogenesis and susceptibility is crucial for developing newer diagnostic and therapeutic strategies for this life threatening infection. In this study, we aimed to analyze the gene expression levels of important cytokines in melioidosis patients and establish useful correlates with disease biomarkers compared to cases of sepsis infection caused by other pathogens and healthy individuals. A Qiagen common human cytokines array, profiling the gene expression of 84 important cytokines by real time quantitative polymerase chain reaction (RT qPCR) was used. We analyzed 26 melioidosis cases, 5 healthy controls and 10 cases of sepsis infection caused by other pathogens. Our results showed a consistent up regulated expression of interleukins; IL4, IL17A, IL23A, IL24, interferons; IFNA1, IFNB1, Tumor necrosis factor (TNF) super family; TNFSF4, Transforming growth factor (TGF) superfamily; bone morphogenetic protein 3,6 (BMP3, BMP6), TGFB1, other growth factors; macrophage colony stimulating factor (M-CSF), C-fos induced growth factor (FIGF) and platelet derived growth factor alpha polypeptide (PDGFA) in melioidosis patients compared to other sepsis cases, irrespective of comorbidities, duration of fever/clinical symptoms and antibiotic treatment. Our findings indicate a dominant Th2 and Th17 type cytokine responses, suggesting that their dysregulation at initial stages of infection may play an important role in disease pathogenesis. IL1A, IL1B and IL8 were significantly down regulated in septicaemic melioidosis patients compared to other sepsis cases. These differentially expressed genes may serve as biomarkers for melioidosis diagnosis, as targets for therapeutic intervention and help us understand immune response mechanisms.

IMPORTANCE

Melioidosis is a life threatening infectious disease caused by a soil-associated gram-negative bacterium, *B. pseudomallei*. Melioidosis is endemic in Southeast Asia and northern Australia; however, the global distribution of *B.pseudomallei* and the disease burden of melioidosis is still poorly understood. Melioidosis is difficult to treat as *B.pseudomallei* is intrinsically resistant to many antibiotics and requires a long course of antibiotic treatment. Mortality rate remains high in endemic areas with reoccurrence being common. Therefore, it is imperative to diagnose the disease at an early stage and provide vital clinical care to reduce the mortality rate. With limitations in treatment and lack of a vaccine, it is crucial to study the immune response mechanisms to this infection to get a better understanding of disease pathogenesis and susceptibility. Therefore, this study aimed to analyze the gene expression levels of important cytokines to establish useful correlation for diagnostic and therapeutic purposes.

INTRODUCTION

Melioidosis is a life threatening infectious disease and is endemic in Southeast Asia and northern Australia (1). A recent report estimates melioidosis disease burden to be 165,000 cases per year (2). Lack of awareness of melioidosis disease among physicians and lack of diagnostic methods contribute to underreporting in many endemic countries. Infection is suspected to be acquired mainly via skin during exposure to soil and contaminated water. Nevertheless inhalation of aerosolized bacteria during extreme weather events such as rainfall and storms has also been reported (2, 3). The disease is strongly associated with comorbidities such as diabetes mellitus, chronic kidney disease, thalassemia, immunosuppression and excessive

73 alcohol intake (1, 4, 5). A broad spectrum of clinical presentations ranging from acute
74 fulminant septicemia to chronic localized abscesses are reported for melioidosis (5).
75 Early diagnosis and appropriate antibiotic treatment plays a crucial role in preventing
76 mortality and recurrence. Advancement of new immunodiagnostic methods and
77 therapeutic strategies is important for disease management of melioidosis, given the
78 lack of vaccines and limitations in drug treatment (3).

79 Studying the host immune responses to infection is crucial for understanding disease
80 pathogenesis, susceptibility and immune correlates of protection (3). Cytokines are
81 vital immune modulators that regulate and determine the nature of immune responses
82 to an infection (6). Activation of leukocytes and cytokine networks are prominent
83 features of inflammation and the septic response (7). Pro- and anti-inflammatory
84 cytokines play a critical role in regulating overall immune responses and in
85 establishing homeostasis, and their dysregulation is instrumental in triggering disease
86 progression and severity (8). Hence a detailed study of the cytokine cascade events at
87 the transcriptome level during an infection is useful to understand disease
88 pathogenesis and susceptibility. Although cytokine cascade events following
89 *B.pseudomallei* infection have been studied in several animal models (7, 9-13), data
90 on human host mRNA expression levels of cytokines is limited. Pro-inflammatory
91 cytokines such as IL8, IL6, IL12, IL18, IL15, IFN γ , TNF α , IL1 β , anti-inflammatory
92 cytokines such as IL4 and several other chemokines have been implicated in disease
93 outcome during the early acute phase of *B.pseudomallei* infection (7, 14-16). While
94 individual cytokines have been investigated in previous studies, the profiling of entire
95 cytokine networks is necessary to comprehensively understand specific immune
96 response pathways and thereby the pathophysiology of melioidosis. Such a profile
97 may also help identify disease biomarkers with therapeutic implications.

We have successfully established a nation-wide surveillance system in Sri Lanka which has resulted in finding more confirmed cases of melioidosis (unpublished data). In this study we aimed to analyze the gene expression profiles of important human cytokines in Sri Lankan melioidosis patients to further understand the immune response mechanisms during melioidosis and establish useful correlates with disease biomarkers.

METHODS

Patient enrollment

Nationwide active surveillance for melioidosis was established in multiple state and private hospitals throughout Sri Lanka, with ethics approval from the Ethics Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka and the Office of Human Research Protection (OHRP), United States Army Medical Research and Material Command (USAMRMC). Patients fitting the clinical case definition of melioidosis i.e. febrile illness for more than 5 days, pneumonia, septic arthritis, skin lesions, septicaemia, lung, soft tissue or deep abscess were recruited for initial screening for melioidosis. Blood, pus and other patient specimens were collected for bacterial cultures and serum samples were collected for indirect haemagglutination (IHA) antibody test. Any positive bacterial cultures were further screened and confirmed as *B.pseudomallei* by PCR. All samples for the study were collected between September 2014 and April 2016.

Patients who were culture positive for *B. pseudomallei* and / or had high antibody titers (>640) by the IHA test were recruited for our study and classified as positive cases of melioidosis. Culture and PCR positive samples were considered as confirmed cases of melioidosis. Samples with an antibody titre of >640 by IHA testing were

considered as probable cases of melioidosis. At the time of recruitment all melioidosis patients were undergoing antibacterial treatment.

We also recruited healthy donors and patients fitting the clinical definition of severe sepsis/septic shock (as per the 2012 WHO guidelines for sepsis management) who were negative for *B. pseudomallei*, as negative controls for our gene expression profiling study (17).

Bacterial culture and identification

Primary isolation of *B. pseudomallei* was done at the admitting hospital using conventional culture techniques for blood, sputum, pus and other specimens. Bacterial isolates that were oxidase positive, gentamicin-resistant and gram-negative bacilli were forwarded to the reference laboratory in Colombo where they were sub-cultured to establish pure growth and maintained at -70°C in 15% brain heart infusion (BHI) glycerol for subsequent definitive tests. Bacteria were resuscitated by subculture onto 5% blood agar and incubated for 24 h at 37°C to give single colony growth for all subsequent tests.

Real time PCR assay for confirmation of *B.pseudomallei*

A single colony of *B. pseudomallei* grown on blood agar from patients sample was re-suspended in ultrapure water. The suspension was heated at 95°C for 10 min and centrifuged at $13500 \times g$ to pellet the cell debris. The supernatant was used as the template for all subsequent PCR assays. Real time PCR assay was done for gene targets of the lpxO, YLF and BTFC gene clusters using the primers and methods described previously (18, 19).

IHA antibody testing

145 Antibody testing against *B.pseudomallei* antigen was performed using an in-house
146 method adapted from Alexander et al, 1970 (20). Antigen was prepared from heat
147 killed culture supernatant of a Sri Lankan *B.pseudomallei*, strain BPs7. A 1/80 diluted
148 antigen preparation was used to sensitize sheep erythrocytes. Serum samples were
149 heat inactivated at 56°C for 30 mins and tested by serial dilution from 1/10 to
150 1/10,240 with sensitized sheep erythrocytes and the reciprocal of the highest dilution
151 at which hemagglutination occurred was recorded as end point titer (20).

152 **Sample collection and processing**

153 10 ml of whole blood was collected from patients/volunteers after written informed
154 consent, of which 7ml were collected into B.D vacutainer mononuclear cell
155 preparation tubes (catalog 362761) for lymphocyte purification. The lymphocytes
156 were purified using the Ficoll fractionation method as per manufacturer's instructions
157 and lysed with RLT buffer (Qiagen RNeasy mini kit-catalog 74104), homogenized
158 and stored at -80°C for total RNA extraction.

159 **Total RNA extraction and cDNA synthesis**

160 Total RNA was extracted from the stored cell lysate samples using the Qiagen
161 RNeasy mini kit (catalog no:74104) as per manufacturer protocol. RNA extracted
162 from 0.6 million PBMC's was used for cDNA synthesis as the standard for all
163 samples analyzed by RT-qPCR. cDNA was synthesized using Qiagen First strand kit
164 (catlog330401) as per the manufacturer recommended instructions. The synthesized
165 cDNA samples were stored at -20°C until further use.

166 **Real Time qPCR and gene expression analysis**

Qiagen human common cytokines RT² Profiler PCR array (catlog PAHS-021Z) was used for this study. The PCR reaction and thermal profile recommended by the manufacturer were followed. 26 melioidosis cases (identified as confirmed or probable cases), 10 other bacterial sepsis cases (negative for *B.pseudomallei*) and 5 healthy negative controls were analyzed by RT-qPCR.

Data Analysis

The relative gene expression ratio, for measuring the change in expression level of a gene was calculated by delta delta CT method (21) as per manufacturer recommendations. The data was normalized using actin beta as the reference housekeeping gene. Statistical analysis was done by Welch's T-test using SAS PROC MIXED, version 9.4. P<0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

A total of 26 cases of melioidosis were analyzed of which 23 were confirmed cases (culture positive), and 3 were probable cases (high antibody titre positive). A majority (n=23) of melioidosis cases had associated comorbidities, and diabetes was the most common comorbidity (n=17) in this study. Out of 23 confirmed cases of melioidosis 16 were classified as septicaemic or bacteriaemic.

The differential expression pattern of interleukins (IL), interferons (IFN), tumor necrosis factor (TNF) super family, transforming growth factor (TGF) super family and other growth factors was significant in melioidosis patients compared to other bacterial sepsis infection cases and healthy controls. Adiponectin, C1Q and collagen domain containing (ADIPOQ) and family with sequence similarity 3, member B

(FAM3B) were significantly down regulated in other bacterial sepsis infection cases compared to healthy controls (fig 1).

Gene expression profile of interleukins

Our study reveals up regulated expression of IL10, IL1B, IL1RN (interleukin 1 receptor antagonist), IL27 and IL8 in melioidosis patients compared to healthy controls (Table 1, Fig 1). This is in agreement with a study by Weirsinga et al, 2007 reporting increased mRNA expression of inflammatory response genes such as IL1 β , IL6, IL15, IL10, IL4, IFN γ and TNF α in melioidosis patients when compared to healthy controls (7). In our study, IL16, IL17A, IL23A and IL24 were down regulated while IL10, IL1B and IL8 were up regulated in other bacterial sepsis infection cases compared to healthy controls (Fig 1). IL16, IL17A, IL17B, IL1RN, IL22, IL23A, IL24, IL27, IL3 and IL4 were all up regulated in melioidosis patients compared to other bacterial sepsis infection cases (Table 2, Fig 1). Particularly, IL17A, IL3 and IL4 showed high levels of gene expression. Previously, expression profiling of interleukins in response to *B.pseudomallei* infection has been extensively studied in several animal models, showing upregulated expression of interleukins such as IL1 β , IL6, IL10 and IL12 within 72 hours of infection (9-13, 15). Elevated levels of expression of IL6, IL8, IL12, IL15 and IL18 was also observed in the plasma of melioidosis patients (15, 16).

IL17A, a pro-inflammatory cytokine which mediates inflammatory responses and induces production of other cytokines, is particularly expressed at very high levels in melioidosis patients (including septicaemic and diabetic cohorts) compared to other sepsis infections (Fig 1- 2). Additionally, IL-22 a widely regarded Th17 cytokine, also shows upregulated expression in melioidosis patients compared to other sepsis cases.

IL17 and other Th17 cytokines are linked to the response against extra-cellular bacteria, pathogenesis of diverse autoimmune and inflammatory diseases, as their dysregulated expression can lead to uncontrolled inflammatory responses (22, 23). IL-17 is also implicated in excessive tissue damage by stimulating the production of many other cytokines including granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), TGF- β , TNF- α , thus contributing to inflammatory pathology (8). IL23, a key mediator of inflammation has also been reported to show upregulated mRNA expression during *B.pseudomallei* infection, implicating its role in pathogenic host immune responses (24). Anti-IL17 and anti-IL23 therapeutic agents have shown to be effective in several immune-mediated inflammatory diseases (23, 25). IL27, implicated in regulating B and T cell activity, has been reported to be significantly elevated in melioidosis patients compared to healthy controls and over production of IL27 plays a major role in pathogenesis of sepsis and shock (26). IL27 has also been identified as a potential sepsis biomarker and a candidate in successful therapeutic intervention (27, 28). As our results show consistent upregulated expression of IL17, IL23 and IL27, their role in melioidosis disease progression and therapeutic use should be further investigated.

Our study revealed greater than 3 fold upregulation of IL4, IL13, IL17A, IL17B, IL22, IL23A, IL24 and IL27 in the diabetic melioidosis cohort (n=17) compared to other bacterial sepsis cases (Fig 2). Diabetes, a risk factor for infectious diseases, may play a role in neutrophil and T-cell dysfunction, possibly mediated by altered glucose metabolism and oxidative stress (29). Studies on diabetic cohorts (mice and human) of melioidosis infection shows excessive neutrophil infiltration and impaired inflammatory and Th1 cytokine responses, leading to increased susceptibility of diabetic individuals to melioidosis (10, 30). IL4, a key regulator of humoral and

239 adaptive immunity, functions as an anti-inflammatory cytokine decreasing production
240 of Th1 cells and related pro-inflammatory cytokines. Our findings show upregulation
241 of IL4 and closely related anti-inflammatory cytokine IL13, in the melioidosis cohort
242 compared to other sepsis cases, which is suggestive of inflammatory responses being
243 dysregulated. Upregulated IL4 expression has been reported in melioidosis patients
244 and acute melioidosis animal models (7, 9).

245 Our findings also show significant upregulation of IL17A, IL17B, IL23A whereas
246 IL1A, IL1B and IL8 were down regulated in septicaemic melioidosis cohort (n=16)
247 compared to other sepsis cases (Fig 1). Downregulation of IL1A, IL1B, IL6, IL8 and
248 IL21 in the early acute phase melioidosis cohort (<15 days fever/clinical symptoms)
249 compared to other sepsis cases (Fig 3) was also seen, indicating IL1A, IL1B and IL8
250 as potential markers during the early stages of inflammation and being correlated with
251 disease severity. A study using a human lung epithelial cell line showed that IL8
252 production upon *B.pseudomallei* infection was lower than cells infected with other
253 gram negative bacteria which correlates with our findings (14). Increased level of
254 plasma IL6 and IL8 concentration, being associated with disease severity and
255 mortality have also been reported (15). Immunosuppression in melioidosis patients
256 correlating to mortality, associated with up regulated interleukin-1R-associated-
257 kinase-M expression, leading to a strong decrease in capacity to release pro-
258 inflammatory cytokines such as IL1B, TNF α and IL8, after *ex-vivo* stimulation with
259 LPS or *B.pseudomallei*, has been reported (31). Downregulation of IL1B upon
260 *B.pseudomallei* infection compared with avirulent *B.thailandensis* in infected lung
261 epithelial cells has also been reported, suggesting host response evasion (32). In our
262 findings, we also see an upregulation of ILRN, a natural inhibitor of pro-
263 inflammatory effects of IL1A, IL1B, in melioidosis patients compared to other

bacterial sepsis cases. Thus IL1 and IL8 which are key mediators of inflammation and early innate immune responses, may serve as candidate early diagnostic markers and indicators of disease severity.

Our findings reveal an upregulated Th2, Th17 cytokine response and a down regulated Th1 cytokine response, with associated comorbidities such as diabetes playing a key role in pathogenesis and severity through dysregulated cytokine responses.

Gene expression profile of interferons

Interferon A5 (IFNA5) was down regulated in melioidosis patients compared to healthy controls (Table 1, Fig 1). Interferon B1 (IFNB1) was significantly down regulated in other sepsis infection cases compared to healthy controls (Fig 1). Interferon A1(IFNA1) and IFNB1 showed upregulation whereas IFNA5 was down regulated in the melioidosis patients compared to other sepsis infection cases (Table 2, Fig 1).

Elevated expression of interferon gamma (IFN γ), a pro-inflammatory cytokine, has been reported in human host and animal models of *B. pseudomallei* infection during the early stages (7, 11, 12, 16). Our findings did not show any significant upregulation of IFN γ in melioidosis cases compared to healthy controls or other bacterial sepsis cases, possibly due to our samples being collected at latter stages while undergoing antibiotic treatment. However we did see a significant upregulation in the diabetic melioidosis cohort compared to other sepsis cases of infection (Fig 2). Interferon mediated responses have been reported as the most dominant pathway, with class I and II interferons being prominent in melioidosis and tuberculosis infections (33). Our study shows an upregulated expression of INFA1 and INFB1 in melioidosis

patients (including diabetic and septicemic cohorts) compared to other sepsis infections (Fig 1,2). Interferons α and β , both belonging to class I interferons, play a major role in innate immune responses. Dysregulated type I IFN production results in a damaging cascade of cell death, inflammation, and immunological host responses that can lead to tissue injury and disease progression (34). Studies have shown type I IFN responses as a striking characteristic of TB infection and that lack of development of Th1 immunity in response to *M. tuberculosis* appears to be associated with increased induction of type 1 IFNs, leading to better bacterial survival and host evasion (35). Furthermore a study also reported that type 1 IFNs suppresses IL-1 production, providing cellular basis for the anti-inflammatory effects, as well as pro-bacterial functions of type I IFNs during *M. tuberculosis* infection (36). Our findings show a similar response, as we see a dominant type I IFN production and a fairly submissive IFN γ and related Th1 cytokines production in melioidosis patients. This data supports our findings of significant downregulated expression of IL1A, IL1B in melioidosis patients compared to other sepsis cases. Type I interferons are also considered as mediators of endotoxic shock and sepsis induced by gram negative bacteria, with IFNB and IFNAR1 deliberated as therapeutic targets (37, 38). Thus further investigation is required to understand the expression of class I interferons in relation to pathogenesis of melioidosis and its role in diagnostic and therapeutic intervention.

Gene expression profile of TNF superfamily

Our findings reveal an upregulation of TNF α an important pro-inflammatory cytokine, in melioidosis cases compared to healthy controls (Table 1, Fig 1). Several studies have reported the upregulated expression of TNF α during melioidosis in

312 human host and animal models of infection (7, 11, 12, 39). Elevated plasma
313 concentrations of TNF α have been correlated with disease severity and mortality in
314 septicaemic melioidosis patients (40).

315 CD40 ligand (CD40LG); which plays a major role in B-cell activation and
316 development and pro-inflammatory cytokines and Lymphotoxin alpha (LTA) were
317 down regulated in the melioidosis cohort compared to healthy controls (Table 1, fig
318 1). CD40L has been considered as an important mediator of sepsis, implicated in
319 platelet-mediated activation and accumulation of neutrophils during inflammation
320 (41, 42).

321 Tumor necrosis factor (ligand) superfamily 14 (TNFSF14); which plays a major role in
322 T-cell proliferation, Tumor necrosis factor (ligand) superfamily 4 (TNFSF4); which is
323 responsible for Th2 cell differentiation, and Tumor necrosis factor (ligand) superfamily 8
324 (TNFSF8); implicated in blocking Th1 responses were up regulated in the melioidosis
325 cohort compared to other sepsis infection cases (Table 1, Fig 1). TNFSF4 was
326 consistently upregulated in melioidosis patients compared to other sepsis cases
327 irrespective of factors like duration of clinical symptoms, antibiotic treatment and
328 comorbidities such as diabetes (fig 1-3). Upregulated expression of TNFSF4 (also
329 known as OX40L), has been observed in cases of polymicrobial sepsis and
330 autoimmune disease and has been correlated to disease severity and mortality (43).

331 Studies have also shown that upregulated expression of TNFSF4 promoted T cell
332 proliferation, increased expression in CD4+ T cells and production of Th2 cytokines
333 such as IL4 (44, 45). It has also been postulated as a specific biomarker in therapeutic
334 interventions for treatment of sepsis/ septic shock and other autoimmune diseases
335 (43). Tumor necrosis factor (ligand) superfamily14 (TNFSF14), otherwise known as
336 LIGHT plays a major role in systemic immune response, particularly in long term

survival of memory Th1 and Th2 cells (46). Tumor necrosis factor (ligand) superfamily 8 (TNFSF8), or CD30L, is reportedly expressed in Th2 cells and suppresses Th1 responses (46). These findings once again suggest an inclination for dominant Th2 responses during the disease progression of melioidosis.

Gene expression profile of TGF β superfamily

Bone morphogenetic protein 6 (BMP6), inhibin beta A (INHBA) and transforming growth factor beta 1 (TGFB1) showed significant upregulation in melioidosis patients compared to healthy individuals (Table 1, Fig 1). BMP3 was down regulated in other sepsis infection cases compared to healthy controls (Fig 1). BMP3, BMP4, BMP6, growth differentiation factor 2 (GDF2), INHBA, TGFB1 expressed up regulated expression in melioidosis patients compared to other sepsis cases, with BMP4 and GDF2 showing high level of gene expression (Table 2 Fig 1).

High level expression of BMP3, BMP6, TGFB1 and TGFB2 was observed in septicaemic melioidosis cohort compared to other bacterial sepsis cases (Fig 1). TGFB2 was expressed at high level in the early acute phase (<15 days of fever/clinical symptoms) melioidosis cohort compared to sepsis control (Fig 3).

TGF β was upregulated during melioidosis infection, with increased levels being correlated to severe melioidosis in human hosts (47). Our study revealed a consistent upregulation of TGFB1 in melioidosis patients compared to other sepsis cases irrespective of factors like duration of clinical symptoms, antibiotic treatment and comorbidities such as diabetes (fig 1-3). An experimental murine model of melioidosis, revealed that an inhibition of TGF- β with a selective TGF- β antibody had a protective effect, with reduction in inflammation, reduced bacterial load and organ damage, thus indicating the role of TGF- β in pathogenesis of melioidosis (47).

Several other studies have also shown the crucial role of TGF- β in immune regulation, where it induces Foxp3, a master regulator of Tregs in naive T cells, with suppression of pro-inflammatory cytokines such as IFN γ and enhanced production of anti-inflammatory cytokines (48, 49). It has also been identified as an inducer of T17 cell differentiation (48, 50). These studies further support our findings of increased Th17 cytokine production and suppression of Th1 cytokines in melioidosis patients.

BMP3 and BMP6 were consistently up regulated in melioidosis patients compared to other sepsis cases irrespective of factors like duration of clinical symptoms, antibiotic treatment and comorbidities such as diabetes (Fig 1-3). BMP's play a major role in formation and repair of bone and cartilage, cell proliferation, differentiation and apoptosis (51). INHBA over expression has been associated with increased cell proliferation and poor disease outcome in several types of carcinomas (52, 53). Further studies are needed to elucidate the mechanisms of BMP signaling pathways and INHBA expression in relation to pathogenesis of melioidosis.

Gene expression profile of Growth factors

Platelet-derived growth factor alpha polypeptide (PDGFA) was upregulated significantly in melioidosis patients compared to healthy individuals (Table 1, Fig 1). PDGFA, thrombopoietin (THPO), ciliary neurotrophic factor (CNTF), macrophage colony stimulating factor (M-CSF or CSF1), C-fos induced growth factor or vascular endothelial growth factor D (FIGF) showed upregulation in melioidosis cases compared to other sepsis infection cases (Table 2, Fig 1). A down regulated expression in THPO was observed in other sepsis cases compared to healthy individuals (Fig 1).

384 PDGF is an important growth factor that plays a crucial role in blood vessel formation
385 (angiogenesis) and regulates cell growth and differentiation. THPO stimulates the
386 production and differentiation of megakaryocytes, thus regulating platelet production.
387 FIGF plays an active role in angiogenesis and vascular endothelial cell growth (54).
388 Increased expression of PDGF is seen in severe bacterial infections, implicating the
389 role of angiogenic factors in endothelial dysfunction leading to disease pathogenesis
390 (54). PDGF has also been suggested as a biomarker of sepsis, related to vascular
391 endothelial damage (55). Our findings also agree with these reports as we see an
392 increased expression of growth factors, which play a role in endothelial function.

393 A down regulated expression of leukemia inhibitory factor (LIF); an IL6 class
394 cytokine that inhibits cell differentiation and a similar cytokine OSM (oncostatin M),
395 was observed in the early acute phase melioidosis cases (<15 days fever/clinical
396 symptoms) compared to other sepsis cases (Fig 3). M-CSF or CSF1, FIGF and
397 PDGFA were consistently up-regulated in melioidosis patients compared to other
398 sepsis cases irrespective of factors like duration of clinical symptoms, antibiotic
399 treatment and comorbidities such as diabetes (Fig 1-3). Studies with experimental
400 mice models of melioidosis have revealed a upregulation of mRNA for macrophage
401 colony stimulating factor (CSF1 or M-CSF), granulocyte macrophage colony
402 stimulating factor (CSF2 or GM-CSF), granulocyte colony stimulating factor (CSF3
403 or G-CSF) at day 3 post infection, correlating with peak bacterial load and extensive
404 infiltration of leucocytes (56). Colony stimulating factors are glycoproteins, necessary
405 for the survival, proliferation and differentiation of hematopoietic progenitor cells of
406 the myeloid and erythroid lineage. M-CSF enhances the survival and activation of
407 cells of the monocyte lineage, while GM-CSF and G-CSF increases accumulation
408 and activation of both neutrophils and macrophages (56). While colony-stimulating

factors play a crucial role in innate immune responses and host defense, their high level of expression during melioidosis may instead contribute to disease pathogenesis.

Limitations of the study

The main limitation of our study was that the melioidosis patient samples were collected after start of antibiotic treatment which may affect immunocompetant cells, which in turn affects the cytokine profiles studied here. Studies have shown that antibiotics like meropenem exert an immunomodulatory effect, affecting the production of some cytokines in PBMC's (57). This may have been the main reason, as to why we could not see any significant differential expression of some key inflammatory response cytokines such as IFN γ . Duration of clinical symptoms ranged from >10 days to >90 days and duration of antibiotics treatment ranged from 3 days to >30 days at the time of blood collection for all the melioidosis samples. Since our sample collection was nationwide, duration between patient identification/disease confirmation and sampling was substantial due to logistical issues. Thus, due to varying and the wide range of acute phase in each of the samples analyzed and less number of samples with ≤ 15 days of fever/clinical symptoms duration (n=5) we could not see any statistically significant differential expression of some of the inflammatory response genes involved in early innate immune responses. However, our results showed a consistent up regulated expression of interleukins; IL4, IL17A, IL23A, IL24, interferons; IFNA1, IFNB1, TNF superfamily; TNFSF4 (OX40L), TGF superfamily; BMP3, BMP6, TGFB1; other growth factors; CSF1, FIGF and PDGFA in melioidosis patients compared to other sepsis cases, irrespective of comorbidities, duration of fever/clinical symptoms and antibiotic treatment, indicating their

differential expression during melioidosis infection. Our findings suggest a domination of Th2 and Th17 type responses during disease pathogenesis of melioidosis.

As diabetes was seen as a major comorbidity in our experimental cohort, we analyzed our data to see if there was any significant differential expression between diabetic melioidosis cases and non-diabetic melioidosis cases. The gene expression pattern between these two groups were comparable and we could not find any statistically significant differential expression, indicating that the differential expression was largely due to melioidosis infection (Tables S1 and S2).

Conclusion

Our study revealed differential gene expression of key cytokines involved in human host responses that can distinguish melioidosis cases from sepsis infections caused by other pathogens and healthy individuals. Low level of expression of key inflammatory mediators; IL1A, IL1B and IL8 were seen in melioidosis patients in early acute phase and with septicaemia compared to other sepsis infection cases. These findings indicate that differentially expressed genes should be validated during different stages of infection for their potential as disease biomarkers for diagnostic purposes and monitoring disease progression. Our results also show an elevated expression of Th17 cytokines such as IL17, IL22 and TGF β which act as an inducer of Th17 cytokines. Th2 cytokines such as IL3, IL4 and IL13 were also upregulated along with type I interferons and TNFSF cytokines, which are known to be inducer's of Th2 cytokines and suppressors of Th1 responses. These results may indicate a dominant Th2 and Th17 type cytokine responses, suggesting that their dysregulation may play an important role in disease pathogenesis and progression. IL17, IL23 and IL27, already implicated in therapeutic intervention of several inflammatory diseases should be

456 further investigated for their role in disease progression and therapeutic approaches in
457 melioidosis.

458 Our future studies shall be aimed at studying gene expression profiles in early and late
459 acute phases of melioidosis to evaluate candidate genes which can serve as disease
460 and diagnostic biomarkers in different stages of infection. Based on these biomarkers
461 if the antibiotic treatment regime can be adjusted it would bring benefits to the
462 patients by reducing the hospital stay. We would expand our studies further, with a
463 larger sample size in each category of sample type, focusing on specific immune
464 response genes showing differential expression, to further understand their role in
465 disease pathogenesis, susceptibility and severity associated with major comorbidities
466 such as diabetes.

467

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REFERENCES

1. **Cheng AC.** 2010. Melioidosis: advances in diagnosis and treatment. Current opinion in Infectious Diseases **23**:554-559.
2. **Limmathurotsakul D, Golding N, Dance DAB, Messina JP, Pigott DM, Moyes CL, Rolim DB, Bertherat E, Day NPJ, Peacock SJ, Hay SI.** 2016. Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. Nature Microbiology **1**.
3. **Patel N, Conejero L, Reynal MD, Easton A, Bancroft GJ, Titball RW.** 2011. Development of vaccines against *Burkholderia pseudomallei*. Frontiers in Microbiology **2**.
4. **Wiersinga WJ, Curie BJ, Peacock SJ.** 2012. Melioidosis. The New England Journal of Medicine **367**.

- 503 5. **Currie BJ, Ward L, Cheng AC.** 2010. The Epidemiology and Clinical Spectrum of
504 Melioidosis: 540 Cases from the 20 Year Darwin Prospective Study. Plos
505 Neglected Tropical diseases **4**.
- 506 6. **Borish LC, Steinke JW .** 2003. Cytokines and chemokines. Journal of allergy and
507 clinical immunology **111**.
- 508 7. **Wiersinga WJ, Dessing MC , Kager PA, Cheng AC, Limmathurotsakul D, Day**
509 **NP, Dondorp AM, van der Poll T, Peacock SJ.** 2007. High-Throughput mRNA
510 Profiling Characterizes the Expression of Inflammatory Molecules in Sepsis
511 Caused by *Burkholderia pseudomallei*. Infection and Immunity **75**:3074–3079.
512
- 513 8. **Chaudhry H, Zhou J , Zhong Y, Ali MM, Mcguire F, Nagarkatti PS and**
514 **Nagarkatti M.** 2013. Role of Cytokines as a Double-edged Sword in Sepsis. In
515 Vivo **27**:669–684.
- 516 9. **Chin CY, Monack DM, Nathan S.** 2010. Genome wide transcriptome profiling of
517 a murine acute melioidosis model reveals new insights into how *Burkholderia*
518 *pseudomallei* overcomes host innate immunity. BMC Genomics **11**.
- 519 10. **Hodgson KA, Govan BL, Walduck AK, Ketheesan N, Morrisa JL.** 2013.
520 Impaired Early Cytokine Responses at the Site of Infection in a Murine Model of
521 Type 2 Diabetes and Melioidosis Comorbidity. Infection and Immunity **81**:470–
522 477.
- 523 11. **Ulett GC, Ketheesan N, Hirst RG.** 2000. Cytokine Gene Expression in Innately
524 Susceptible BALB/c Mice and Relatively Resistant C57BL/6 Mice during
525 Infection with virulent *Burkholderia pseudomallei*. Infection and Immunity
526 **68**:2034–2042.
- 527 12. **Ulett GC, Ketheesan N, Clair TW, McElnea CL, Barnes JL, Hirst RG.** 2002.
528 Analogous Cytokine Responses to *Burkholderia pseudomallei* Strains Contrasting

- 529 in Virulence Correlate with Partial Cross-Protection in Immunized Mice.
 530 Infection and Immunity **70**:3953–3958.
- 531 13. **Wiersinga JW, Dessing MC, vander Poll T.** 2008. Gene-expression profiles in
 532 murine melioidosis. *Microbes and Infection* **10**:868-877.
- 533 14. **Utaisincharoen P, Anuntagool N, Arjcharoen S, Lengwehasatit I,**
 534 **Limposuwan K, Chaisuriya P, Sirisinha S.** 2004. *Burkholderia pseudomallei*
 535 stimulates low interleukin-8 production in the human lung epithelial cell line
 536 A549. *Clin Exp Immunol* **138**:61-65.
- 537 15. **Friedland JS, Suputtamongkol Y, Remick DG, Chaowagul W, Strieter RM,**
 538 **Kunkel SL, White NJ, Griffin GE.** 1992. Prolonged elevation of Interleukin8 and
 539 Interleukin6 concentration in plasma and of leukocyte Interleukin 8 mRNA
 540 levels during septicemic and localized pseudomonas pseudomallei infection.
 541 *Infection and Immunity* **60**:2402-2408.
- 542 16. **Lauw FN, Simpson AJH, Prins JM, Smith MD, Kurimoto M, van Deventer**
 543 **SJH, Speelman P, Chaowagul W, White NJ, van der Poll T.** 1999. Elevated
 544 Plasma Concentrations of Interferon (IFN)-g and the IFN-g-Inducing Cytokines
 545 Interleukin (IL)-18, IL-12, and IL-15 in Severe Melioidosis. *The Journal of*
 546 *infectious diseases* **180**:1878–1885.
- 547 17. **Dellinger RP, Levy MW, Rhodes A, Annane D, Gerlach H, Opal SM, Sevransky**
 548 **JE, et al.** 2013. Surviving Sepsis Campaign: International Guidelines for
 549 Management of Severe Sepsis and Septic Shock: 2012. *Critical care medicine* **41**.
- 550 18. **Tuanyok A, Auerbach RK, Brettin TS, Bruce DC, Munk AC, Detter JC,**
 551 **Pearson T, Hornstra H, Sermswan RW, Wuthiekanun V, Peacock SJ, Currie**
 552 **BJ, Keim P, Wagner DM.** 2007. A Horizontal Gene Transfer Event Defines Two
 553 Distinct Groups within *Burkholderia pseudomallei* That Have Dissimilar
 554 Geographic Distributions. *Journal of Bacteriology* **189**:9044–9049.

- 555 19. **Merritt A, Inglis TJJ, Chidlow G, Harnett G.** 2006. PCR Based identification of
556 *Burkholderia pseudomallei*. Rev Inst Med trop S Paulo **48**:239-244.
- 557 20. **Alexander AD, Huxsoll DL, Warner AR, Shepler V, Dorsey A.** 1970.
558 Serological Diagnosis of Human Melioidosis with Indirect Hemagglutination and
559 Complement Fixation Tests. Applied Microbiology **20**: 825-833.
- 560 21. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using
561 real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods
562 **25**:402-408.
- 563 22. **Onishi RM and Gaffen SL.** 2010. Interleukin-17 and its target genes:
564 mechanisms of interleukin-17 function in disease. Immunology **129**:311–321.
565
- 566 23. **Bosmann M and Ward PA .** 2012. Therapeutic potential of targeting IL-17 and
567 IL-23 in sepsis. Clinical and Translational Medicine **1**.
- 568 24. **Kulsantiwon P, Pudla M, Boondit J, Wikraiphat C, Dunachie SJ, Chantratita**
569 **N, Utaisinchaoen P.** 2015. *Burkholderia pseudomallei* induces IL-23 production
570 in primary human monocytes. Med Microbiol Immunol doi:10.1007/s00430-
571 015-0440-z.
- 572 25. **Gaffen SL, Jain R, Garg AV and Cua DJ.** 2014. IL-23-IL-17 immune axis:
573 Discovery, Mechanistic Understanding, and Clinical Testing. Nat Rev Immunol
574 **14**:585-600.
- 575 26. **Rinchai D, Khaenam P, Kewcharoenwong C, Buddhisa S, Pankla R,**
576 **Chaussabel D, Bancroft GJ, and Lertmemongkolchai G.** 2012. Production of
577 interleukin 27 by human neutrophils regulates their function in response to
578 bacterial infection. Eur J Immunol **42**:3280–3290.
- 579 27. **Wong HR, Lindsell CJ, Lahni P, Hart KW and Gibot S.** 2013. Interleukin-27 as a
580 sepsis diagnostic biomarker in critically ill adults. Shock **40**:382–386.

- 581 28. **Scicluna BP and van der Poll T** . 2012. Interleukin-27: a potential new sepsis
582 biomarker exposed through genome-wide transcriptional profiling. *Critical Care*
583 **16**.
- 584 29. **Hodgson K, Morris J, Bridson T, Govan B, Rush C and Ketheesan N**. 2014.
585 Immunological mechanisms contributing to the double burden of diabetes and
586 intracellular bacterial infections. *Immunology* **144**:171–185.
- 587 30. **Morris J, Williams N, Rush C, Govan B, Sangla K, Norton R, Ketheesan N**.
588 2012. *Burkholderia pseudomallei* Triggers Altered Inflammatory Profiles in a
589 Whole-Blood Model of Type 2 Diabetes-Melioidosis Comorbidity. *Infection and*
590 *Immunity* **80**:2089–2099.
- 591
- 592 31. **Wiersinga WJ, van't Veer C, van den Pangaart PS, Dondorp AM, Day NP,**
593 **Peacock SJ, van der Poll T**. 2009. Immunosuppression associated with
594 interleukin-1R-associated-kinase-M upregulation predicts mortality in Gram-
595 negative sepsis (melioidosis). *Crit Care Med* **37**:569-576.
- 596 32. **Wongprompitak P, Sirisinha S and Chaiyaroj SC** . 2008. Differential Gene
597 Expression Profiles of Lung Epithelial Cells Exposed to *Burkholderia*
598 *pseudomallei* and *Burkholderia thailandensis* during the Initial Phase of Infection.
599 *Asia pacific journal of allergy and immunology* **26**:59-70.
- 600 33. **Koh GCKW, Schreiber MF, Bautista R, Maude RR, Dunachie S, et al**. 2013.
601 Host Responses to Melioidosis and Tuberculosis Are Both Dominated by
602 Interferon-Mediated Signaling. *PLoS ONE* **8**:e54961.
- 603 34. **Reddy MA, Park JT, Natarajan R**. 2013. Epigenetic Modifications in the
604 Pathogenesis of Diabetic Nephropathy. *Semin Nephrol* **33**:341–353.
- 605 35. **Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, Barry CE,**
606 **Freedman VH and Kaplan G**. 2001. Virulence of a *Mycobacterium tuberculosis*

- 607 clinical isolate in mice is determined by failure to induce Th1 type immunity and
608 is associated with induction of IFN-gamma. PNAS **98**:5752–5757.
- 609 36. **Mayer-Barber KD, Andrade BB, Barber DL, Hieny S, Feng CG, Caspar P,**
610 **White S, Gordon S and Sher A.** 2011. Innate and adaptive interferons suppress
611 IL-1 α and IL-1 β production by distinct pulmonary myeloid subsets during
612 Mycobacterium tuberculosis infection. Immunity **35**:1023–1034.
- 613 37. **Mahieu T and Libert C.** 2007. Should We Inhibit Type I Interferons in Sepsis.
614 Infection and Immunity **75**:22–29.
- 615 38. **Weighardt H, Kaiser-Moore S, Schlautko'tter S, Rossmann-Bloeck T,**
616 **Schleicher U, Bogdan C, Holzmann B.** 2006. Type I IFN Modulates Host
617 Defense and Late Hyperinflammation in Septic Peritonitis. The Journal of
618 Immunology **177**:5623-5630.
- 619
- 620 39. **Sim SH, Liu Y, Wang D, Novem V, Sivalingam SP, et al.** 2009. Innate Immune
621 Responses of Pulmonary Epithelial Cells to *Burkholderia pseudomallei* Infection.
622 PLoS ONE **4**:e7308.
- 623 40. **Suputtamongkol Y, Kwiatkowski D, Dance DAB, Chaowagul W and White N**
624 **J.** 1992. Tumour necrosis factor in septicemic melioidosis. The Journal of
625 Infectious Diseases **165**:561-564.
- 626 41. **Rahman M, Zhang S, Chew M, Ersson A, Jeppsson B, Thorlacius H.** 2009.
627 Platelet-derived CD40L (CD154) mediates neutrophil upregulation of Mac-1 and
628 recruitment in septic lung injury. Ann Surg **250**:783-790.
- 629 42. **Wang X, Qin W, Sun B.** 2014. New strategy for sepsis: Targeting a key role of
630 platelet-neutrophil interaction. Burns & Trauma **2**.
- 631 43. **Karulf M, Kelly A, Weinberg AD and Gold JA.** 2010. OX40L Regulates
632 Inflammation and Mortality in the Innate Immune Response to Sepsis. J Immunol
633 **185**:4856–4862.

- 634 44. **Lei W, Zeng D, Zhu C, Liu G, Zhang X, Wang C, Wang Q, Huang J.** 2014. The
635 upregulated expression of OX40/OX40L and their promotion of T cells
636 proliferation in the murine model of asthma. *J Thorac Dis* **6**:979-987.
- 637 45. **Linton PJ, Bautista B, Biederman E, Bradley ES, Harbertson J, Kondrack**
638 **RM, Padrick RC, Bradley LM.** 2003. Costimulation via OX40L Expressed by B
639 Cells Is Sufficient to Determine the Extent of Primary CD4 Cell Expansion and
640 Th2 Cytokine Secretion In Vivo. *J Exp Med* **197**:875–883.
- 641 46. **Croft M, Duan W, Choi H, Eun S, Madireddi S, Mehta A.** 2012. TNF superfamily
642 in inflammatory disease: translating basic insights. *Trends Immunol* **33**:144–
643 152.
- 644
- 645 47. **Weehuizen TAF, Wieland CW, van der Wind GJW, Duitman JW, Boon L, Day**
646 **NPJ, Peacock SJ, van der Poll T and Wiersinga WJ.** 2012. Expression and
647 Function of Transforming Growth Factor β in Melioidosis. *Infection and*
648 *Immunity* doi:10.1128/IAI.05534-11:1853–1857.
- 649 48. **Yoshimura A, Wakabayashi Y and Mori T.** 2010. Cellular and molecular basis
650 for the regulation of inflammation by TGF- β . *J Biochem* **147**:781–792.
- 651 49. **Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G and Wahl SM.**
652 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+
653 regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*
654 **198**:1875–1886.
- 655 50. **Korn T, Oukka M, Kuchroo V and Bettelli E.** 2007. Th17 cells: effector T cells
656 with inflammatory properties. *Semin Immunol* **19**:362–371.
- 657 51. **Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M et al.** 2014. Bone
658 Morphogenetic Protein (BMP) signaling in development and human diseases.
659 *Genes and diseases* **1**:87-105.

- 660 52. **Seder CW, Hartojo W, Lin L, Silvers AL, Wang Z, Thomas DG et al.** 2009.
661 Upregulated INHBA Expression May Promote Cell Proliferation and Is
662 Associated with Poor Survival in Lung Adenocarcinoma. *Neoplasia* **11**:388–396.
- 663 53. **Seder CW, Hartojo W, Lin L, Silvers AL, Wang Z, Thomas DJ et al.** 2009.
664 INHBA Overexpression Promotes Cell Proliferation and May Be Epigenetically
665 Regulated in Esophageal Adenocarcinoma. □ *Journal of Thoracic Oncology* **4**.
- 666 54. **Mankhambo LA, Banda DL, IPD Study Group: Jeffers G, White SA, Balmer P,**
667 **Nkhoma S, Phiri H, Molyneux EM, Hart CA, Molyneux ME, Heyderman RS,**
668 **Carrol ED.** 2010. The role of angiogenic factors in predicting clinical outcome in
669 severe bacterial infection in Malawian children. *Critical Care* **14**.
- 670
- 671 55. **Khan MF and Rajendrum R .** 2015. New markers in sepsis. *Anaesth Pain &*
672 *intensive Care* **19**:108-111.
- 673 56. **Barnes JL, Ulett GC, Ketheesan N, Clair T, Summers PM, Hirst RG.** 2001.
674 Induction of multiple chemokine and colony-stimulating factor genes in
675 experimental *Burkholderia pseudomallei* infection. *Immunology and Cell*
676 *Biology* **79**:490–501.
- 677 57. **Djaldetti M, Nachmias N, Bessler H.** 2016. The effect of antibiotics on cytokine
678 production by mononuclear cells and the cross-talk with colon cancer cells.
679 *Journal of Pharmacy & Pharmacognosy Research* **4**:134-143.

680

681

682 **TABLES**

683 **Table 1. Cytokines showing significant differential expression in PBMC's of**
684 **meliodosis patients (n=26) compared to healthy negative controls (n=5)**

Gene	Gene description	Relative expression ratio [95% CI]	P value	
IL1B	Interleukin 1 beta	2.504 [1.229 - 5.100]	0.0135	PB
IL1RN	Interleukin 1 receptor antagonist	1.62 [1.023 - 2.564]	0.0403	MC
IL8	Interleukin 8	2.953 [1.394 - 6.257]	0.0062	,
IL10	Interleukin 10	2.257 [1.180 - 4.319]	0.0158	peri
IL27	Interleukin 27	4.022 [1.632 - 9.915]	0.0039	phe
INFAS	Interferon alpha 5	0.189 [0.037 - 0.961]	0.0454	ral
TNF	Tumour necrosis factor	2.248 [1.082 - 4.670]	0.0315	blo
CD40LG	CD40 ligand	0.502 [0.336 - 0.752]	0.0023	od
LTA	Lymphotoxin alpha	0.327 [0.190 - 0.565]	0.0008	mo
BMP6	Bone morphogenetic protein 6	2.946 [1.470 - 5.904]	0.0075	non
INHBA	Inhibin beta A	6.07 [2.652 - 13.891]	0.0002	ucle
TGFB1	Transforming growth factor 1	1.634 [1.121 - 2.383]	0.0126	ar
PDGFA	Platelet derived growth factor alpha polypeptide	2.86 [1.444 - 5.667]	0.0066	cell
			699	s;
			700	CI,

701 confidence interval; Relative expression ratio >1.5 indicates upregulation and ≤0.5
702 indicates downregulation in the experimental group compared to control group; Gene
703 targets showing significant differential expression where P-value <0.05

704

705 **Table 2. Cytokines showing significant differential expression in PBMC's of**
706 **melioidosis patients (n=26) compared to other sepsis cases (n=10)**

Gene symbol	Gene description	Relative expression ratio [95% CI]	P value
IL3	Interleukin 3	9.38 [1.773 - 49.626]	0.0107
IL4	Interleukin 4	6.024 [1.153- 31.479]	0.0344
IL16	Interleukin 16	1.896 [1.152 - 3.121]	0.0157
IL17A	Interleukin 17 alpha	16.32 [3.193 - 83.421]	0.0017
IL17B	Interleukin 17 beta	2.939 [1.486 - 5.811]	0.003
IL1RN	Interleukin 1 receptor antagonist	1.747 [1.091-2.796]	0.0216
IL22	Interleukin 22	3.022 [1.207 - 7.565]	0.0206
IL23A	Interleukin 23 alpha	2.792 [1.329 -5.866]	0.0092
IL24	Interleukin 24	2.991 [1.240 -7.214]	0.0173
IL27	Interleukin 27	3.089 [1.203 - 7.932]	0.0206
INFA1	Interferon alpha 1	4.034 [1.358 -11.984]	0.014
INFA5	Interferon alpha 5	0.2 [0.057 - 0.704]	0.0152
INFB1	Interferon beta 1	3.206 [1.056 - 9.735]	0.0407
TNFSF4	Tumour necrosis factor super family 4	2.349 [1.167 -4.728]	0.0202
TNFSF8	Tumour necrosis factor super family 8	1.606 [1.004 -2.571]	0.0484
TNFSF14	Tumour necrosis factor super family 14	2.353 [1.171 - 4.728]	0.0186
BMP3	Bone morphogenetic protein 3	5.305 [2.319 - 12.135]	0.0003
BMP4	Bone morphogenetic protein 4	18.765 [1.479 -238.054]	0.0271
BMP6	Bone morphogenetic protein 6	2.776 [1.214 - 6.344]	0.0192
GDF2	Growth differentiation factor 2	11.112 [1.105 -111.704]	0.0421
INHBA	Inhibin beta A	4.635 [1.205 -17.822]	0.0282

707		TGFB1	Transforming growth factor 1	2.006 [1.374 - 2.931]	0.0007
708		PDGFA	Platelet derived growth factor alpha polypeptide	2.317 [1.065 - 5.038]	0.0357
709					
710	PB	THPO	Thrombopoietin	4.213 [1.042 - 17.040]	0.0441
711	MC,	CNTF	Ciliary neurotrophic factor	2.222 [1.023 - 4.829]	0.0441
712	perip	CSF1	Colony stimulating factor 1	2.456 [1.451 - 4.156]	0.0017
713	heral	FIGF	C-fos induced growth factor	3.912 [1.561 - 9.802]	0.0049

714 blood mononuclear cells; CI, confidence interval; Relative expression ratio >1.5
715 indicates upregulation and ≤ 0.5 indicates downregulation in the experimental group
716 compared to control group; Gene targets showing significant differential expression
717 where P-value <0.05

718

FIGURES

Fig 1: Relative differential gene expression of cytokines in melioidosis patients compared to patients with sepsis infection due to other pathogens and healthy negative controls. Significant relative gene expression changes in PBMC's from melioidosis patients (n=26) and septicaemic melioidosis patients (n=16) compared to sepsis controls (n=10) and healthy controls (n=5). Expression levels were normalized against beta actin as the reference house keeping gene. Relative expression ratio >1.5 considered as upregulation and ≤ 0.5 was considered as downregulation. * indicates relative expression ratio is significantly different ($P<0.05$)

Fig 2: Relative differential gene expression of cytokines in diabetic melioidosis patients compared to patient with sepsis infection due to other pathogens and healthy negative controls. Relative gene expression in PBMC's from diabetic melioidosis patients (n=17), compared to sepsis controls (n=10) and healthy controls (n=5). Expression levels were normalized against beta actin as the reference house keeping gene. Relative expression ratio >1.5 was considered as upregulation and ≤ 0.5 considered as downregulation. * indicates relative expression ratio is significantly different ($P<0.05$)

Fig 3: Relative differential gene expression of cytokines in melioidosis patients with respect to duration of fever/clinical symptoms and antibiotics treatment, compared to patients with sepsis infection due to other pathogens. Relative gene expression in PBMC's from melioidosis patients with ≤ 15 days of fever (n=5), melioidosis patients with >15 days of fever (n=21), melioidosis patients with ≤ 15 days of treatment with antibiotics (n=14), melioidosis patients with >15 days of treatment with antibiotics (n=8), compared to sepsis controls (n=10). Expression

743 levels were normalized against beta actin as the reference house keeping gene.

744 Relative expression ratio >1.5 was considered as upregulation and ≤ 0.5 considered as

745 downregulation. * indicates relative expression ratio is significantly different ($P < 0.05$)

746

747

Table 1: Primer details* for Gene expression analysis

Primer Name	Forward Sequence	Reverse Sequence	Product Size
GAPDH2	TGACAACTTTGGTATYCGTGGAAGG	AGGCAGGGATGATGTTCTGGAGAG	134 bp
18srRNA	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC	69 bp
PLCE1	GCCCAAAGCAAGTGGAAGG	TCTTCACCTGGGTAAACATGC	700 bp
IL8	CAGAGACAGCAGAGCACACA	GGCAAACTGCACCTTCACA	158 bp
MICB	CACCCAGGCTGCAGTTCCT	CGGGAGTCTGAGGTACGAGAA	88 bp
PSMB8	GATCTCCAGAGCTCGCTTTA	GTTACCCGTAAGGCACTAA	200 bp
CCL5	CCATGAAGGTCTCCGCGGCAC	CCTAGCTCATCTCCAAAGAG	361 bp
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	88 bp
IL18	GCTTGAATCTAAATTATCAGTC	CAAATTGCATCTTATTATCATG	335 bp
DNMT1A	ACCGCTTCTACTTCCTCGAGGCCTA	GTTGCAGTCCTCTGTGAACACTGTG G	335 bp
DNMT3B	AATGTGAATCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT	191 bp
HDAC1	CCAAGTACCACAGCGATGAC	TGGACAGTCCTCACCAACG	110 bp
HDAC2	TGAAGGAGAAGGAGGTGCGAA	GGATTTATCTTCTTCCTTAACGTCTG	124 bp
DNMT3A	CGTTGGCATCCACTGTGAATGA	TTACACACACGCAAAATACTCCTT	551bp
IFN γ	CCAACGCAAAGCAATACATGA	CCTTTTTCGCTTCCCTGTTTTA	79 bp
TNF α	GGA GAA GGG TGA CCG ACT CA	CTG CCC AGA CTC GGC AA	70 bp
IL1 β	GCAAGGGCTTCAGGCAGGCCGCG	GGTCATTCTCCTGGAAGGTCTGTGG GC	96 bp
IL4	ACTTTGAACAGCCTCACAGAG	TTGGAGGCAGCAAAGATGTC	74 bp
IL15	GTCTTCATTTTGGGCTGTTTCAGT	CCTCACATTCTTTGCATCCAGATTCT	316 bp
TLR2	GGGTCATCATCAGCCTCTCC	AGGTCACTGTTGCTAATGTAGGTG	181 bp
TLR4	CAGAGTTGCTTTCAATGGCATC	AGACTGTAATCAAGAACCTGGAGG	282 bp

PSME2	GGGAATGAGAAAGTCCTGTCC	TCAATCTTGGGGATCAGGTG	113 bp
IL12	CCAAGAAGCTGCAGCTGAAG	TGGGTCTATTCCGTTGTGTC	355bp
HDAC4	GAGAGACTCACCTTCCCG	CCGGTCTGCACCAACCAAG	240bp
PSMA5	AAGCCCATGTTGCTTTTGGG	GGCGAACGGACATAGGCTAA	112bp
PSMB2	AGAGGGCAGTGGAAGCTCCTT	AGGTTGGCAGATTCAGGATG	72bp
HMGB1	ACATCCAAAATCTTGATCAGTTA	AGGACAGACTTTCAAAATGTTT	122bp
HLADMB	ATGTGAAATCCTTTGGAGTCCCA	GGCATCTTTACAGAGCAGAGCAT	145bp

748 *GAPDH and 18srRNA primers are the house keeping genes. PLCE1 primer pair tested to
749 amplify 700bp genomic region of PLCE1 is used as genomic DNA control.

750

751 Table 2. mRNA expression in PBMC's of melioidosis patients (n=30) compared to other
 752 sepsis cases (n=10) and healthy negative controls (n=10)

753

	Melioidosis vs Healthy controls		Melioidosis vs Sepsis controls		Sepsis cases vs Healthy controls	
Gene	Relative expression ratio [95% CI]	P value	Relative expression ratio [95% CI]	P value	Relative expression ratio [95% CI]	P value
HMGB1	0.83[0.444,1.539]	0.5324	0.26[0.132,0.510]*	0.0005	3.18[1.539,6.578]*	0.0036
IL6	1.56[0.670,3.623]	0.2912	0.89[0.360,2.189]	0.7867	1.76[0.710,4.344]	0.2079
IL8	1.65[0.727,3.733]	0.2187	0.43[0.169,1.080]	0.0699	3.86[1.411,10.540]*	0.0114
IL1 β	1.33[0.554,3.205]	0.5005	0.77[0.258,2.320]	0.6254	1.72[0.516,5.738]	0.3545
IFN γ	1.34[0.826,2.189]	0.2174	0.88[0.496,1.572]	0.6522	1.52[0.800,2.899]	0.1864
TNF α	1.22[0.705,2.124]	0.4573	0.81[0.408,1.598]	0.5173	1.52[0.741,3.100]	0.2365
IL15	1.31[0.751,2.296]	0.3254	0.57[0.258,1.279]	0.1610	2.29[1.025,5.103]*	0.0443
IL4	1.78[0.115,27.531]	0.3533	4.09[1.178,14.173]*	0.0366	0.44[0.065,2.942]	0.2329
TLR2	1.16[0.666,2.034]	0.5844	0.44[0.196,1.009]	0.0522	2.62[1.182,5.803]*	0.0212
TLR4	0.97[0.532,1.777]	0.9247	0.37[0.141,0.974]*	0.0448	2.62[1.025,6.700]*	0.0450
MICB	0.95[0.533,1.699]	0.8607	0.33[0.183,0.582]*	0.0006	2.92[1.534,5.545]*	0.0026
HLADMB	0.84[0.502,1.396]	0.4814	0.69[0.396,1.204]	0.1809	1.21[0.682,2.156]	0.4897
PSMB2	0.88[0.513,1.519]	0.6403	0.25[0.121,0.508]*	0.0008	3.56[1.708,7.420]*	0.0021
PSME2	1.24[0.763,1.998]	0.3757	0.38[0.195,0.726]*	0.0061	3.28[1.684,6.389]*	0.0017
PSMB8	0.83[0.456,1.519]	0.5356	0.27[0.131,0.565]*	0.0014	3.06[1.443,6.480]*	0.0060
PSMA5	0.75[0.421,1.328]	0.3086	0.84[0.412,1.706]	0.6102	0.89[0.448,1.778]	0.7301
DNMT1A	0.66[0.376,1.171]	0.1494	0.51[0.211,1.213]	0.1171	1.31[0.542,3.169]	0.5224
DNMT3A	0.68[0.416,1.097]	0.1087	0.68[0.356,1.300]	0.2284	0.99[0.533,1.849]	0.9793
DNMT3B	1.07[0.636,1.814]	0.7770	0.22[0.088,0.539]*	0.0040	4.94[1.948,12.503]*	0.0031
HDAC1	0.78[0.556,1.095]	0.1441	0.50[0.353,0.719]*	0.0006	1.55[1.059,2.267]*	0.0266
HDAC2	0.68[0.377,1.212]	0.1799	0.28[0.126,0.642]*	0.0048	2.38[1.047,5.403]*	0.0398
HDAC4	0.99[0.588,1.652]	0.9549	0.64[0.322,1.270]	0.1880	1.54[0.786,3.025]	0.1911

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755 PBMC, peripheral blood mononuclear cells; CI, confidence interval; Relative expression ratio
756 >1 indicates up regulation and ≤ 0.5 indicates down regulation in the experimental group
757 compared to control group; * indicates statistically significant differential expression where
758 P-value < 0.05 , P values calculated by paired t-tests

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Figure 1. Relative expression of genes involved in immune response and epigenetic regulation in melioidosis patients compared to patients with sepsis infections caused by other pathogens and healthy controls. Statistically significant differential expression of genes in PBMC's from melioidosis patients (n=30), septicaemic melioidosis patients (n=18) compared to sepsis controls (n=10). Significant differential expression was not observed among melioidosis patients compared to healthy controls (n=10) while significant differential expression was observed among sepsis controls and the healthy controls. Expression levels were normalized against 18srRNA. Relative expression ratio >1.5 considered as up regulation and ≤ 0.5 considered as down regulation, with $P < 0.05$ considered statistically significant.

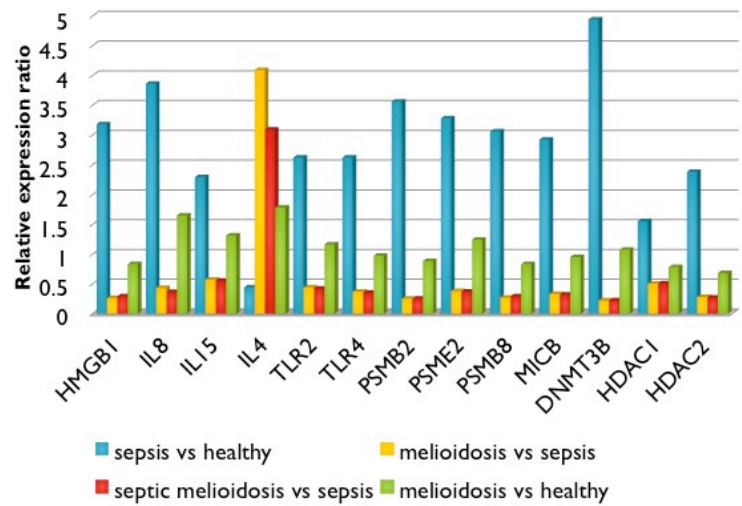


Figure 2. Relative expression of genes involved in immune responses and epigenetic regulation in melioidosis patients compared to patients with sepsis infections caused by other pathogens, in relation to duration of fever/clinical symptoms and antibiotics treatment. Differential gene expression in PBMC's from melioidosis patients with ≤ 15 days of fever (n=4), melioidosis patients with >15 days of fever (n=25), melioidosis patients with ≤ 15 days of treatment with antibiotics (n=15), melioidosis patients with >15 days of treatment with antibiotics (n=12) compared to sepsis controls (n=10), did not change due to the duration of fever or duration of treatment with antibiotics. Expression levels were normalized against 18srRNA. Relative expression ratio >1.5 considered up regulated and ≤ 0.5 considered as down regulation, with $P < 0.05$ considered statistically significant.

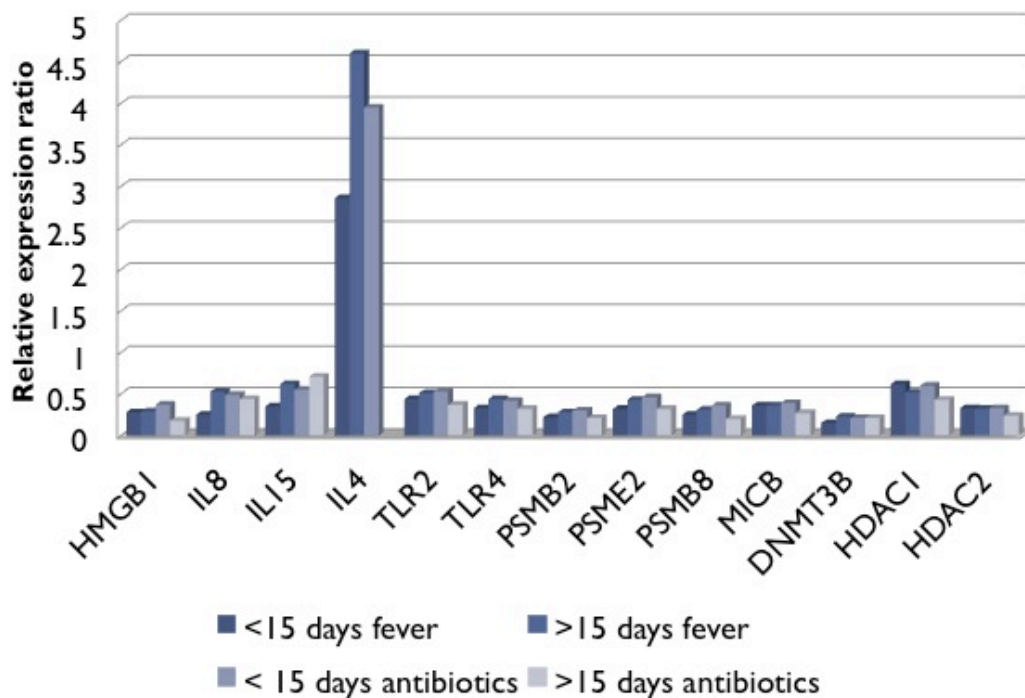
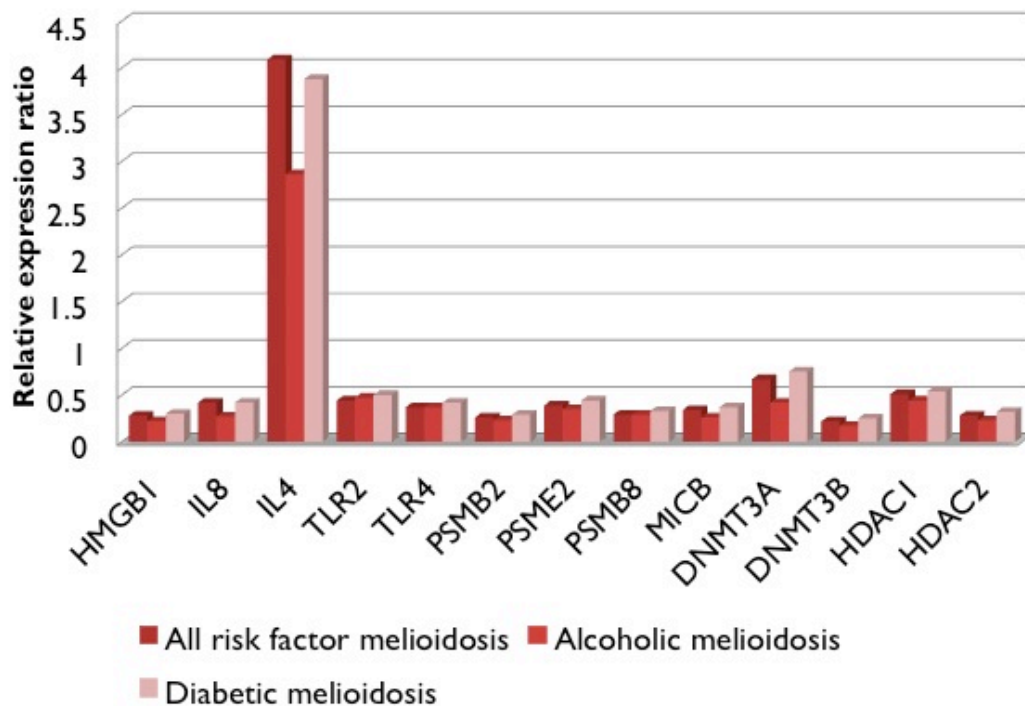


Figure 3. Relative expression of genes involved in immune responses and epigenetic regulation in melioidosis patients compared to patients with sepsis infections caused by other pathogens, in relation to associated comorbidities.

Differential gene expression in PBMC's from melioidosis patients with risk factors (n=27), alcoholic melioidosis patients (n=8), diabetic melioidosis patients (n=20), compared to sepsis controls (n=10), did not change among patients presented with different risk factors for melioidosis. Expression levels were normalized against 18srRNA. Relative expression ratio >1.5 was considered as up regulated and ≤ 0.5 was considered as down regulated, with $P < 0.05$ considered statistically significant.



819 **S1: List of gene targets investigated**

Abbreviated name	Full Name	Biological Role
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Involved in several metabolic processes such as glycolysis
18SrRNA	18S ribosomal RNA	Structural RNA and basic component of eukaryotic cells
PLCE1	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1	Involved in intracellular responses involving cell growth and differentiation
IL1 β	Interleukin 1 beta	Pro-inflammatory cytokine, important mediator of the inflammatory response, involved in a variety of cellular activities, including cell proliferation, differentiation and apoptosis.
IL4	Interleukin 4	Anti-inflammatory cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells, decreasing production of Th1 cells. It is a key regulator in humoral and adaptive immunity playing a major role in stimulation of activated B-cell and T-cell proliferation.
IL6	Interleukin 6	Pro-inflammatory cytokine and an anti-inflammatory myokine. It is secreted by T cells and macrophages to stimulate immune response
IL8	Interleukin 8	Chemokine associated with inflammation, induces chemotaxis in target cells, mainly involved in neutrophil recruitment and degranulation.
IL10	Interleukin 10	Anti-inflammatory cytokine involved in immune regulation and inflammation. It down regulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF- κ B activity, and is involved in the regulation of the JAK-STAT signaling pathway.
IL12	Interleukin 12	Pro-inflammatory cytokine involved in the differentiation of naive T cells into Th1 cells. It is involved in stimulation and growth T cells and in production of interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α) from T cells and natural killer (NK) cells. It reduces IL-4 mediated suppression of IFN- γ .
IL15	Interleukin 15	Pro-inflammatory cytokine which regulates T and natural killer (NK) cell activation and proliferation.

IL18	Interleukin 18	Pro-inflammatory cytokine involved in inflammation and cell-mediated immunity along with IL12
CCL5	Chemokine (C-C motif) ligand 5. Also known as RANTES (regulated on activation, normal T cell expressed and secreted).	Chemokine, which is chemotactic for T cells, eosinophils and basophils, plays an active role in recruiting leukocytes into inflammatory sites
IFN γ	Interferon gamma	Pro-inflammatory cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections. It is an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression.
TNF α	Tumor necrosis factor alpha	Pro-inflammatory cytokine involved in systemic inflammation and immune regulation
HMGB1	High mobility group box 1 protein, also known as high-mobility group protein 1 (HMG-1)	Cytokine mediator of inflammation secreted by activated macrophages and monocytes
TLR2	Toll-like receptor 2	Plays a fundamental role in pathogen recognition and activation of innate immunity. This gene is expressed abundantly in peripheral blood leukocytes and mediates host response to gram-positive bacteria.
TLR4	Toll-like receptor 4	Plays a fundamental role in pathogen recognition and activating the innate immune system. It is well-known for recognizing lipopolysaccharide (LPS), a component present in many gram-negative bacteria, thus mediates its host responses
MICB	MHC class I polypeptide-related sequence B	Heavily glycosylated protein which is a ligand for the NKG2D type II receptor. Binding of the ligand activates the cytolytic response of natural killer (NK) cells, CD8 alpha beta T cells, and gamma delta T cells which express the receptor.
PSMB8	Proteasome subunit beta type-8 also known as 20S proteasome subunit beta-5i	Forms a pivotal component for the Ubiquitin-Proteasome System (UPS) involved in protein ubiquitination and subsequent proteolysis and degradation which are important mechanisms in the regulation of the cell cycle, cell growth and differentiation, gene transcription, signal transduction and apoptosis. During the antigen processing for the major histocompatibility complex (MHC) class-I, the proteasome is the major degradation machinery that degrades the antigen and present the resulting peptides to cytotoxic T cells.

PSMB2	Proteasome subunit beta type-2 also known as 20S proteasome subunit beta-4	Forms a pivotal component for the UPS involved in protein ubiquitination and subsequent proteolysis and degradation which are important mechanisms in the regulation of the cell cycle, cell growth and differentiation, gene transcription, signal transduction and apoptosis. It is also involved in processing of class I MHC peptides
PSME2	Proteasome activator complex subunit 2	Process class I MHC peptides
PSMA5	Proteasome subunit alpha type-5 also known as 20S proteasome subunit alpha-5	Process class I MHC peptides
HLADMB	HLA class II histocompatibility antigen, DM beta chain	Plays a central role in the peptide loading of MHC class II molecules by helping to release the CLIP (class II-associated invariant chain peptide) molecule from the peptide binding site, thus playing a major role in MHC class II antigen presentation pathway
DNMT1A	DNA methyltransferase 1A	Enzyme catalyzes the transfer of methyl groups to specific CpG structures in DNA (DNA methylation). Considered to be the key maintenance methyl transferase in mammals. predominantly methylates hemi methylated CpG dinucleotides in the mammalian genome.
DNMT3A	DNA (cytosine-5)-methyl transferase 3A	DNA methyl transferaseresponsible for de novo DNA methylation.
DNMT3B	DNA (cytosine-5-)-methyl transferase 3 beta	DNA methyl transferase responsible for de novo DNA methylation.
HDAC1	Histone deacetylase 1	Class I histone deacetylase, playing a key role in the regulation of eukaryotic gene expression
HDAC2	Histone deacetylase 2	Class I histone deacetylase, playing a key role in transcriptional regulation and regulation of eukaryotic gene expression
HDAC4	Histone deacetylase 4	Class II histone deacetylase, playing a key role in transcriptional regulation and regulation of eukaryotic gene expression

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822 **S2: Primer details for Gene expression analysis**

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Primer Name	Forward Sequence	Reverse Sequence	Product Size
GAPDH ^a	TGACAACTTTGGTATYCGTGGAAGG	AGGCAGGGATGATGTTCTGGAGAG	134 bp
18srRNA ^a	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC	69 bp
PLCE1 ^b	GCCCAAAGCAAGTGGAAAGG	TCTTCACCTGGGTAAACATGC	700 bp
IL8	CAGAGACAGCAGAGCACACA	GGCAAAACTGCACCTTCACA	158 bp
MICB	CACCCAGGCTGCAGTTCCT	CGGGAGTCTGAGGTACGAGAA	88 bp
PSMB8	GATCTCCAGAGCTCGCTTTA	GTTCAACCGTAAGGCACTAA	200 bp
CCL5	CCATGAAGGTCTCCGCGGCAC	CCTAGCTCATCTCCAAAGAG	361 bp
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC	88 bp
IL18	GCTTGAATCTAAATTATCAGTC	CAAATTGCATCTTATTATCATG	335 bp
DNMT1A	ACCGCTTCTACTTCCTCGAGGCCTA	GTTGCAGTCCTCTGTGAACACTGTG G	335 bp
DNMT3B	AATGTGAATCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT	191 bp
HDAC1	CCAAGTACCACAGCGATGAC	TGGACAGTCCTCACCAACG	110 bp
HDAC2	TGAAGGAGAAGGAGGTCGAA	GGATTTATCTTCTTCCTTAACGTCTG	124 bp
DNMT3A	CGTTGGCATCCACTGTGAATGA	TTACACACACGCAAAATACTCCTT	551 bp
IFN γ	CCAACGCAAAGCAATACATGA	CCTTTTTCGCTTCCCTGTTTTA	79 bp
TNF α	GGA GAA GGG TGA CCG ACT CA	CTG CCC AGA CTC GGC AA	70 bp
IL1 β	GCAAGGGCTTCAGGCAGGCCGCG	GGTCATTCTCCTGGAAGGTCTGTGG GC	96 bp
IL4	ACTTTGAACAGCCTCACAGAG	TTGGAGGCAGCAAAGATGTC	74 bp
IL15	GTCTTCATTTTGGGCTGTTTCAGT	CCTCACATTCTTTGCATCCAGATTCT	316 bp
TLR2	GGGTCATCATCAGCCTCTCC	AGGTCACTGTTGCTAATGTAGGTG	181 bp
TLR4	CAGAGTTGCTTTCAATGGCATC	AGACTGTAATCAAGAACCTGGAGG	282 bp

PSME2	GGGAATGAGAAAGTCCTGTCC	TCAATCTTGGGGATCAGGTG	113 bp
IL12	CCAAGAAGCTTGCAGCTGAAG	TGGGTCTATTCCGTTGTGTC	355 bp
HDAC4	GAGAGACTCACCTTCCCG	CCGGTCTGCACCAACCAAG	240 bp
PSMA5	AAGCCCATGTTGCTTTTGGG	GGCGAACGGACATAGGCTAA	112 bp
PSMB2	AGAGGGCAGTGGAATCCTT	AGGTTGGCAGATTCAGGATG	72 bp
HMGB1	ACATCCAAAATCTTGATCAGTTA	AGGACAGACTTTCAAAATGTTT	122 bp
HLADMB	ATGTGAAATCCTTTGGAGTCCCA	GGCATCTTTACAGAGCAGAGCAT	145 bp

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825 ^aGAPDH and 18srRNA primers are house keeping genes. ^bPLCE1 primer pair tested to
826 amplify 700bp genomic region of PLCEI is used as genomic DNA control.

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